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Early Host Damage in the Infection Cycle of *Bdellovibrio bacteriovorus*

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The effects of bdellovibrio infection on host permeability and respiration were investigated by measuring respiration rates and the rate of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis during the course of single infection cycles of *Bdellovibrio bacteriovorus* strain 109 growing on *Escherichia coli* ML 35 (*lac* $i^-z^+y^-$). The data show that among the very early consequences of parasite attack on the host are an increase in permeability and a general disruption of respiratory activity of the host, and it is suggested that both phenomena stem from early damage to host membrane. The rapid onset of damage after inception of the cycle and the failure of streptomycin to prevent the damage indicate that complete penetration of the parasite into the host is not a requirement for the observed effects. The data also show that bdellovibrio does not use host energy-generating mechanisms for its growth and suggest that the parasites may have a search mechanism that permits them, to some degree, to distinguish between infected and uninfected hosts.

The infection cycle of *Bdellovibrio bacteriovorus* has been studied extensively by phase microscopy, microcinematography, and electron microscopy, and by now a qualitative picture of the sequence of events from its attachment to a host to release of progeny upon host lysis has been well established (8, 13). Quantitative information on aspects of the infection cycle is meager and largely confined to data on the kinetics of attachment and penetration of the parasite (15) and to progeny yields from growth on a limited number of hosts in a few nutritional environments (7, 16). The enzymology of the parasite has been the subject of a single paper (10), and little or nothing has been reported on the biochemical events involved in invasion and development of the parasite.

The investigations reported here are concerned with the last-mentioned problem. They grew out of an exploratory experiment in which the endogenous respiration and the stimulation of this respiration by succinate were determined for *B. bacteriovorus* strain 109 and *Escherichia coli* B. Since the parasite did not respire succinate while the host did, it was felt that the measurement of succinate respiration during the course of an infection cycle might provide information on changes that could be ascribed unequivocally to the host. When such an experiment was done, it was found that the potential of the host-parasite complex to respire succinate increased sharply shortly after initiation of infection and then declined with almost equal rapidity.

Two explanations were considered for the observed increase in potential to respire succinate. The first is that during the early phase of an infection cycle bdellovibrios can respire succinate even though the free parasite does not. This possibility had to be considered since Simpson and Robinson (10) showed that *B. bacteriovorus* strain 6-5-S possesses a complete tricarboxylic acid cycle. An alternative explanation is that the rate of succinate permeation limits its rate of respiration by the uninfected host and that infection by bdellovibrios renders the host more permeable. To explore this problem further, a cryptic strain of *E. coli* lacking lactose permease and constitutive for β-galactosidase was chosen as the host. As is shown later, *B. bacteriovorus* has essentially no β-galactosidase.

MATERIALS AND METHODS

Host. *E. coli* ML 35 (*lac* $i^-z^+y^-$) was obtained from the collection of the Department of Bacteriology, The Hebrew University-Hadassah Medical School, Jerusalem, and was maintained on nutrient agar slants. Cell suspensions were prepared from nutrient broth cultures incubated on a rotary shaker at 37 C. Stationary-phase cells were harvested from overnight cultures (16 to 24 hr). To obtain cell suspensions of enhanced respiratory activity, overnight cultures were diluted 1 to 2 with fresh nutrient broth and

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incubated for an additional 1 to 1.5 hr. For convenience, such suspensions will be referred to as "exponential-phase" cells. Cells were harvested by centrifugation at 12,000 × *g* for 5 min and washed once by suspending the pellet in one-half the original volume of tris(hydroxymethyl)aminomethane (Tris) buffer, 10^{-3} M, *p*H 7.5. The washed cells were centrifuged and resuspended in the same buffer to the desired concentration by reference to a standard curve relating viable cell count to turbidity measured with a Klett-Summerson photoelectric colorimeter. Separate standard curves were prepared for stationary and exponential phase cells.

Parasite. *B. bacteriovorus* strain 109, described by Stolp and Starr (14), was used. The culture was maintained by daily transfers using as the host *E. coli* growing in DNB medium [0.8 g of nutrient broth (Difco), 0.5 g of Casamino Acids, 0.1 g of yeast extract (Difco), 0.2 g of $Ca(NO_3)_2$, 0.01 g of $FeSO_4$, 0.01 g of $MnSO_4$, 1,000 ml of water, *p*H 7.6 (9)]. To obtain cells for experimental purposes, 250 ml of DNB medium in a 1-liter Erlenmeyer flask was inoculated with 1 ml of bdellovibrio stock culture and 10 ml of stationary-phase *E. coli*, and the flask was shaken at 30 C for 18 to 24 hr. Before harvesting, cultures were examined microscopically to make sure that essentially all host cells were lysed. At the time of harvesting, the DNB cultures contained 10^9 to 3 × 10^9 bdellovibrio per ml (by turbidity measurements) and fewer than 100 viable *E. coli* per ml (by colony formation on nutrient agar).

The bdellovibrio cultures were centrifuged at 1,500 × *g* for 3 min, to sediment debris which consisted in part of iron and manganese salts that precipitate from the DNB medium, and then at 27,000 × *g* for 20 min to sediment the bdellovibrios. The pellet from the second centrifugation was washed once in 125 ml of 10^{-3} M Tris buffer, *p*H 7.5, and resuspended in the same buffer. The cell suspensions were adjusted to the desired populations by reference to a standard curve relating cell numbers determined by plaque assay to Klett units.

The double-layer technique described by Varon and Shilo (15) was used for the plaque assays. Cell numbers determined in this way corresponded (±20%) to cell numbers determined by direct count in a Petroff-Hauser chamber for bdellovibrio cultures grown as described and harvested within a few hours after complete lysis of the host.

General design of experiments. Experiments were done in an environment in which the host cells served as the exclusive source of nutrients for the parasites, i.e., in 10^{-3} M Tris buffer, *p*H 7.5. A multiplicity of infection (MOI) of two parasites, or greater, per host was used, except when the multiplicity was the experimental variable, to insure that essentially all host cells in an experiment were infected within a brief period after mixing hosts and parasites. Typically, host populations were in the range of 2.5 × 10^9 to 5.0 × 10^9 cells per ml. Experiments were initiated by mixing suspensions of host and parasite (zero time) and incubating at 30 C, either with shaking in a water bath or with aeration in an Oxygraph cell. In the former case, samples of the mixture were removed at appropriate intervals for measurements of respiration rates, rates of hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG), or other parameters.

An organic compound, usually lactose or succinate at 5 μmoles per ml final concentration, was frequently added to the system either at zero time or to samples removed from a mixture at subsequent times. These compounds were not added for nutritional purposes but as indicators to detect and amplify changes occurring in the host cells during bdellovibrio infection. Appropriate controls showed that the compounds were in no way necessary for causing the changes they helped to measure. The control experiments involved incubating parallel mixtures of infected *E. coli* with and without the indicator compound and determining whether a measurement made after adding a pulse of the compound to a portion of the unsupplemented mixture gave the same result as a measurement made at the same elapsed time on the parallel system containing the compound from zero time. Only one set of control data will be presented (*see* Fig. 1), but in all cases the results with the two types of procedures were qualitatively identical and quantitatively very similar.

Presentation of the data. Most of the data are presented as plots of rates [(dx/dt), respiration, or ONPG hydrolysis] as a function of time after mixing host and parasite suspensions. The slopes of the curves obtained are thus representations of the rate of change of rates [d(dx/dt)/dt]. Since, however, the rates (dx/dt) are measures of primary host functions, y (e.g., permeability control or respiratory potential), the slopes of the curves are representations of the rate of change in the hosts during infection, dy/dt.

Measurement of oxygen consumption. Oxygen consumption was measured polarigraphically with an Oxygraph model KM (G.M.E., Middleton, Wis.). The instrument was adjusted so that full-scale deflection on its recorder was equivalent to the uptake of 0.5 μmole of oxygen from a reaction mixture having a total volume of 2.1 ml. The glucose-glucose oxidase reaction in the presence of catalase was used for calibration purposes. All measurements were made in a water-jacketed cell at 30 C containing 2.1 ml of reaction mixture.

In short-term experiments when less than 0.5 μmole of oxygen was consumed (e.g., Tables 1 and 2), the instrument was used in the conventional manner and respiration rates were determined from the slopes of the continuous traces of oxygen concentration recorded.

For intermediate length experiments (30 to 60 min) in which many closely spaced rate determinations were made and in which oxygen consumption far exceeded that initially dissolved in the samples (Fig. 3–6), the reaction mixtures were aerated in the Oxygraph cell between rate measurements by means of a fine stream of air passing through a capillary tube. A typical experiment was started by introducing the final component of the mixture being examined into the cell at zero time, e.g., the parasite suspension. The capillary tube was inserted and the experimental

mixture was both mixed and aerated by the air stream. At 45 sec, the capillary tube was withdrawn; at 60 sec, the instrument was switched on for 5 sec to record the oxygen concentration in the system. The 5-sec measurement was repeated at least twice at successive 15-, 30- or 60-sec intervals thereafter, depending on the rate of oxygen consumption. The contents of the Oxygraph cell were then reaerated to maintain aerobic conditions until 15 sec before the next series of measurements. The data were thus recorded as a series of steps, the heights of which were a measure of the oxygen consumed over the time increment used. Base-line drift was not significant during a 30- to 60-min period.

For experiments of long duration (Fig. 1 and 2), the mixture in the Oxygraph cell was removed at roughly 30-min intervals and replaced with samples from a larger, but otherwise identical, experimental mixture being concurrently shaken in a 30 C water bath. The base line of the instrument was readjusted between samples.

β-Galactosidase activity. The unmasking of the β-galactosidase activity of the cryptic host during infection was followed by measuring the rate of ONPG hydrolysis. Samples of host-parasite system were removed at intervals after mixing and added to the ONPG hydrolysis mixture of Wallenfels (17). The change in optical density at 405 nm was recorded spectrophotometrically, and rates of hydrolysis were determined from the slopes of the curves obtained, which were linear after 15 to 30 sec.

Miscellaneous methods. The procedures of Varon and Shilo (15) were used to measure bdellovibrio attachment to and penetration into their host. The ^{14}C-labeled bdellovibrios were mixed with nonlabeled hosts, and samples of the mixture, removed at intervals, were filtered through 1.2-μm cellulose acetate filters and washed. The amount of label retained on the filter, either without or with pretreatment of the sample being filtered in an Omni-mixer, was the measure of attachment or penetration, respectively.

Glucose was assayed with the glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). Protein contents of cells were determined after a 10-min digestion in sodium hydroxide by the procedure of Lowry, et al. (4) with bovine serum albumin as the standard.

RESULTS

Bdellovibrio infection cycle under the experimental conditions employed. Phase contrast microscopy showed that under the experimental conditions described above, the bdellovibrios exhibited a typical infection cycle. Attachment was observed immediately upon mixing host and parasite suspensions and proceeded rapidly; penetration of some parasites into host cells was seen in about 20 min, and almost all host cells had been converted to spheroplasts by 30 to 45 min. Those bdellovibrios that did not attach to host cells lost motility rapidly, and by 1 hr few, if any, free motile parasites could be seen. At least some of the nonmotile free bdellovibrios were probably nonviable since suspensions of free bdellovibrios shaken at 30 C in 10^{-3} M Tris buffer, *p*H 7.5, showed a progressive decrease in plaque-forming units with time. The onset of host lysis, usually in 2.5 to 3 hr, was signalled by the reappearance of highly motile, free bdellovibrios that had the typical size and appearance of newly released progeny. Lysis was complete in 3.5 to 4 hr.

Quantitative data also confirmed that the infection cycle is normal under the general experimental conditions. An attachment and penetration experiment showed, as Varon and Shilo (15) previously found, that attachment was virtually complete in 30 min and that penetration was first measurable between 10 and 15 min after combining host and parasite suspensions. Finally, a comparison of the number of plaque-forming units at the start and end of an infection cycle showed that multiplication of the parasite had occurred.

Respiration rates of B. bacteriovorus strain 109 and E. coli ML 35. The endogenous respiration rates and protein contents of parasite and host were determined on suspensions prepared from a number of independently grown cultures during the course of these studies (Table 1). On a per-cell

TABLE 1. *Endogenous respiration and protein contents of B. bacteriovorus strain 109 and exponential cells of E. coli ML35*

Determination	*B. bacteriovorus*			*E. coli*		
	O_2 used[a]	Protein[b]	RQ[c]	O_2 used[a]	Protein[b]	RQ[c]
Average	18	0.42	44	27	4.5	5.9
Range	13–27	0.36–0.47		21–36	4.0–4.8	
Standard deviation	±4	±0.05		±6	±0.2	
No. of samples	25	13		10	9	

[a] Expressed as nmoles per min per 10^{10} cells at 30 C.
[b] Expressed as mg per 10^{10} cells.
[c] Respiration quotient, expressed as nanomoles per minute per milligram of protein at 30 C.

basis, the average endogenous respiration rate of *B. bacteriovorus* strain 109 is about 70% that of *E. coli* ML 35 even though its protein content is only about 10% that of its host. The endogenous respiration of suspensions of the separate organisms did not change significantly over a 60-min period of shaking or aeration in the Oxygraph cell at 30 C.

Table 2 presents data on the respiration rates of the host and parasite in the presence of a variety of substrates. The data were obtained with single suspensions of each and are typical of results in several independent experiments. Of the substrates listed, only peptone significantly increased the respiration of the bdellovibrios and the effect was small, about 30%. Yeast extract or casein hydrolysate also stimulated bdellovibrio respiration to about the same extent as peptone.

The respiration of host-free suspensions of *B. bacteriovorus* in the presence of lactose or succinate was measured on many occasions during these studies. Lactose was consistently without effect; succinate, however, occasionally and unpredictably stimulated respiration by 10 to 20%. Because of the special significance of these two compounds in the investigations, cultures of *B. bacteriovorus* were grown on *E. coli* B growing in synthetic medium with either lactose or succinate as the sole carbon source for the host to see whether an enhanced respiration of these compounds could be induced in the parasite. Suspensions of bdellovibrios harvested from these cultures did not have an increased respiration rate on the homologous substrates.

In contrast to the parasite, the host *E. coli* actively respired a variety of compounds at rates of up to 20 times endogenous. Lactose, however, at a concentration of 5×10^{-3} M did not stimulate the respiration of freshly harvested exponential phase cells, a confirmation of the essentially complete impermeability of the host to this substrate. There was no detectable increase in the respiration of such suspensions after being shaken in 5×10^{-3} M lactose-10^{-3} M Tris buffer, *p*H 7.5, at 30 C for 1 hr. Exponential-phase cells stored in this buffer overnight at 4 C and freshly harvested stationary-phase cells usually showed a small increase in respiration rate in the presence of lactose.

TABLE 2. *Respiration of various substrates by B. bacteriovorus strain 109 and by E. coli ML35*[a]

Substrate	*B. bacteriovorus*	*E. coli*
None	19	33
Lactose	18	35
Succinate	17	600
Acetate	20	677
Glucose	20	293
Lactate	20	512
Malate	19	422
Peptone	24	

[a] Values are expressed as nmoles of O_2 per min per 10^{10} cells.

Changes in respiration rates during an infection cycle. Figure 1 shows the respiration rates of an unsupplemented mixture of bdellovibrio and *E. coli* and the effect of lactose on these rates during the course of a complete infection cycle. After a small initial increase and a plateau, the rate of respiration of the unsupplemented system rose steadily from about 1 to 3 hr and reached a maximum of some three times the rate at the start of the cycle. The maximum coincided in time with the onset of lysis of host cells. Lysis was complete in about 4 hr; the respiration rate dropped as progeny were released and reached a value of about two-thirds maximum at 1 hr after the end of the cycle.

The potential of the host-parasite complex to respire lactose showed a much different pattern. Lactose-stimulated respiration increased rapidly over a 25-min period after the start of the cycle. The rapid increase was followed by an almost equally rapid decline, and by 1 hr stimulation of respiration by lactose was only 20% of the maximum effect. By 2 hr, the addition of lactose to the system had no effect on its respiration. In a parallel experiment in which lactose was continuously present in the system from zero time on, the respiration pattern (Fig. 1, dotted line) completely paralleled that observed when lactose was present only at the time of each measurement. It may be concluded that the changes observed occur independently of the presence of lactose.

Figure 2 shows the results of a similar experiment in which succinate was used as the indicator compound. The respiration pattern of the unsupplemented host-parasite system duplicated that observed in the previous experiment. The pattern of succinate-stimulated respiration differed significantly from that observed for lactose in that there was no increase in the potential to respire this compound early in the cycle. Instead, starting at about 10 min after infection, the succinate-stimulated respiration, which was initially high, dropped rapidly, and by 60 min was only 17% of the initial.

Changes in respiratory patterns early in the infection cycle. Closely spaced measurements made over the first 30 min of the cycle showed (Fig. 3) that the respiration rate of the unsupplemented host-parasite complex started to rise at

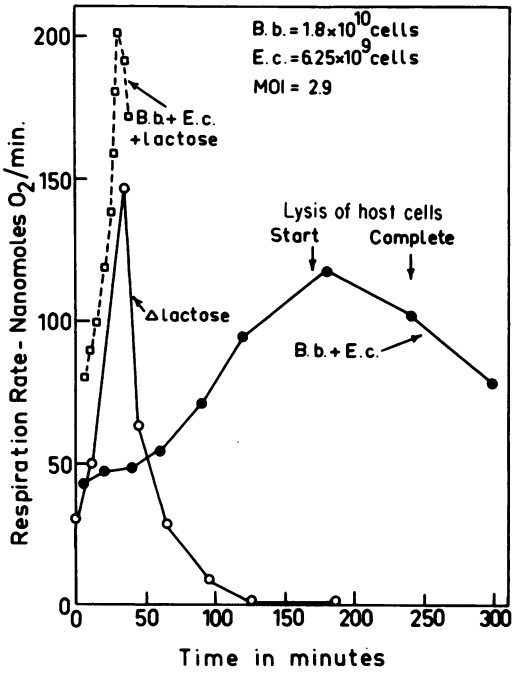

FIG. 1. *Endogenous and lactose respiration patterns during an infection cycle of B. bacteriovorus growing on a lactose cryptic E. coli. Host and parasite suspensions were mixed and shaken at 30 C. Samples (2 ml) were removed at intervals and their respiration rates (●) and the stimulation of these rates by 10 μmoles of lactose (○) were determined. Control (□), respiration rates of the same host-parasite system with lactose continuously present from start. Abbreviations (this and subsequent figures): MOI, multiplicity of infection; B.b., Bdellovibrio bacteriovorus; E.c., Escherichia coli. All rates are for indicated number of starting cells. Host lysis was judged qualitatively by phase microscopy.*

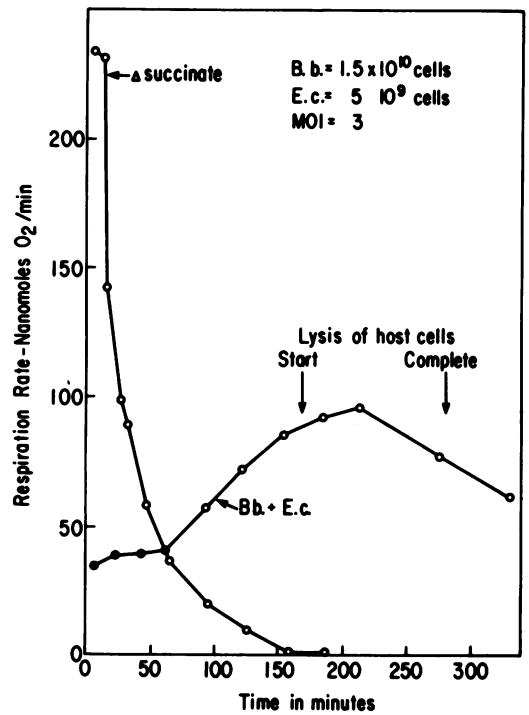

FIG. 2. *Endogenous and succinate respiration patterns during an infection cycle of B. bacteriovorus growing on E. coli. Experimental protocol as in Fig. 1. Symbols: ●, endogenous rates; ○, stimulation of rates on addition of 10 μmoles of succinate.*

about 5 min after mixing and reached a maximum some 15 min later. The changes in rate were small in absolute terms and were overshadowed in comparison to the very marked changes observed in the presence of either indicator compound. The increase was consistently observed, however, and is appreciable in relation to the zero-time respiration rate of the system (Fig. 4). On the average, the increase was about 35%.

Changes in the rate of respiration of lactose were first detected in this experiment between 5 and 10 min after starting the cycle (Fig. 3), and peak potential was reached in 20 min. The angular peak of the lactose respiration rate curve is an artifact of the discontinuous method of measuring rates and the manner of plotting the data. By choosing the proper number of hosts and parasites, it was possible to get a continuous record of the change in oxygen concentration in the

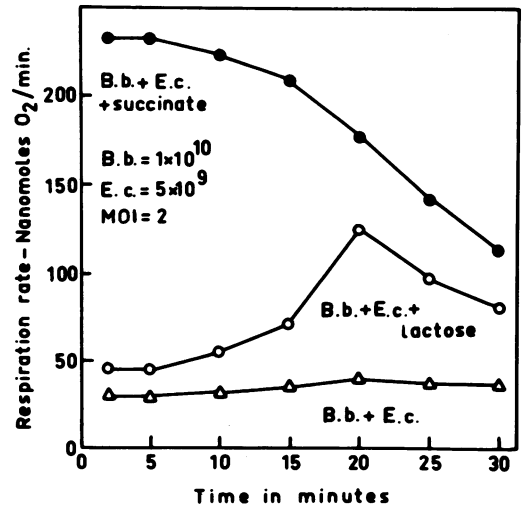

FIG. 3. *Changes in respiratory patterns in early stages of an infection cycle of B. bacteriovorus growing on E. coli: △, endogenous rates, 10^{10} bdellovibrios plus 5×10^9 E. coli; ○, same plus 10 μmoles of lactose; ●, same plus 10 μmoles of succinate.*

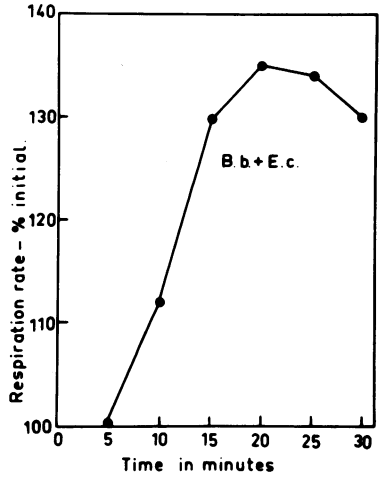

FIG. 4. *Changes in endogenous respiration in early stages of an infection cycle of B. bacteriovorus growing on E. coli. The results plotted are the averages of five experiments done at a constant MOI with host population varied from 2.5 × 10^9 to 2.0 × 10^{10}.*

system over the period of the lactose respiration maximum. The curve had no discontinuities—the maximum rate of lactose respiration was approached by a period of decreasing acceleration and was followed by a period of increasing deceleration of rate.

The ability of the system to respire succinate started to decline at about the time that an increase in the rate of lactose respiration was detectable (Fig. 3), and the rate of this decline was linear with time between 15 and 30 min. In some experiments, there was a small initial rise in the rate of succinate respiration before the onset of decline (e.g., Fig. 13); in other experiments at multiplicities of infection from 10 to 20, the decline was observed immediately after mixing host and parasite.

The effects of several variables on these changes were examined. The time of occurrence of the maximum rate of lactose respiration was, on the average, somewhat later when stationary cells rather than exponential phase cells served as hosts. The rate at the maximum was, however, about the same with both types of hosts at comparable numbers of hosts and parasites.

The maximum rate of lactose respiration attained was a linear function of the number of cells in the system at a fixed multiplicity of infection. At a fixed host population, the pattern of the lactose respiration rate varied in a more complex manner as the multiplicity of infection was varied (Fig. 5). The time of occurrence of the maximum was progressively later as the multiplicity decreased. The magnitude of the maximum respiration rate, corrected for the respiration of the unsupplemented system, increased, however, as the multiplicity was decreased from 16 to 1.6. The data suggest that two processes are occurring concurrently, one resulting in an increased and the other in a decreased potential to respire lactose, with the latter speeded up more than the former as the multiplicity of infection increases.

At a given multiplicity of infection, the decay of the rate of succinate respiration was linear during most of the process (Fig. 6). The slopes of the decay curves increased with an increasing multiplicity of infection. A plot of the decay rate against the multiplicity of infection (insert, Fig. 6) shows a nonlinear relation between these parameters. A plot of the number of bdellovibrios that attach to a fixed number of host cells against the multiplicity of infection, calculated from the data of Varon and Shilo (15), gives a similar curve. The combined data suggest that the decay of succinate respiration is a function of the number of parasites infecting a host.

The respiration of a few other substrates was also followed during the early phases of the infection cycle. With all compounds tested, the respiratory potential decreased markedly and to about the same degree (Table 3). Lactate, glucose, and malate were similar to succinate in that the rate of respiration of these compounds started to decrease almost immediately after mixing host and parasite. Acetate was similar to

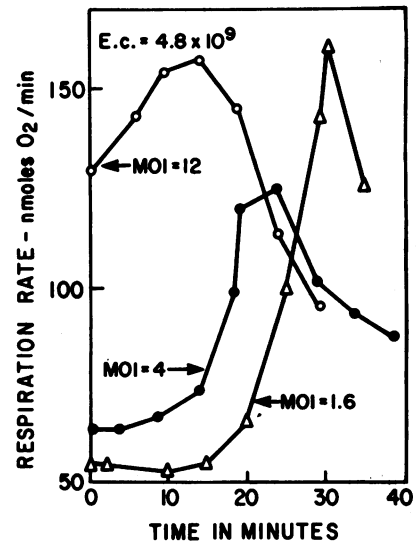

FIG. 5. *Lactose respiration pattern as a function of the multiplicity of infection: 4.8 × 10^9 E. coli and 10 μmoles of lactose plus bdellovibrios to MOI of 12* (○); *4* (●); *and 1.6* (△).

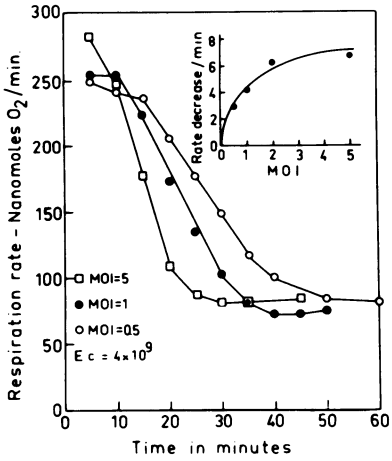

FIG. 6. *Succinate respiration pattern as a function of MOI: 4 × 10⁹ E. coli and 10 μmoles of succinate plus bdellovibrios to MOI of 5 (□); 1 (●); and 0.5 (○). The insert is a plot of the slope of the linear portion of the decay curves as a function of MOI.*

lactose in that the rate of its respiration increased before declining. However, the increase was small, the maximal rate (at 10 min) being some 15% greater than the initial.

Changes in the rate of ONPG hydrolysis during the infection cycle. Although the increase in potential to respire lactose with time after infection can only mean an increased permeability of the host to this compound, the subsequent decrease in respiration rate could have several possible causes: repair of the damage responsible for increased permeability, destruction of β-galactosidase, or damage somewhere in the chain of reactions connecting the products of lactose hydrolysis to oxygen. To decide among these possibilities, the unmasking of β-galactosidase activity in the cryptic host during the infection cycle was observed with ONPG as the substrate (Fig. 7). The lactose respiration potential of the same system was determined concurrently and showed the pattern already described. In contrast, the rate of ONPG hydrolysis continued to rise for about 45 min and then remained more or less constant until the end of the experiment. The final rate of ONPG hydrolysis was approximately 20% greater than that measured with an equal number of *E. coli* broken by sonic oscillation and thus probably represents complete expression of the total β-galactosidase content of the hosts. An equal number of sonically disrupted bdellovibrio hydrolyzed ONPG at a rate of about 7 nmoles per min, approximately 0.001 that of the host rate. Only a trace of β-galactosidase activity was found in supernatant fluids of the host-parasite system at 45 or 60 min after infection. These supernatant fluids were slightly cloudy and the activity measured could have been due to unsedimented cells.

The results clearly show that the decrease of the lactose respiration rate is not due to destruction of β-galactosidase or to its release from the host cells. The results also exclude repair of damage leading to increased permeability as a

TABLE 3. *Effect of bdellovibrio infection on the respiration of various compounds by E. coli*[a]

Compound	Respiration rate at[b]		
	0 min	30 min	Decrease
			%
Succinate	214	75	65
Acetate	90[c]	36	60
Glucose	99	47	53
Lactate	219	75	66
Malate	129	60	54

[a] System: 4 × 10⁹ *E. coli*, 10¹⁰ bdellovibrios, and 10 μmoles of substrate in 2.1 ml of 0.001 M Tris buffer, *p*H 7.5, 30 C.

[b] Corrected for respiration in absence of substrate.

[c] Respiration rate at 10 min (maximum rate).

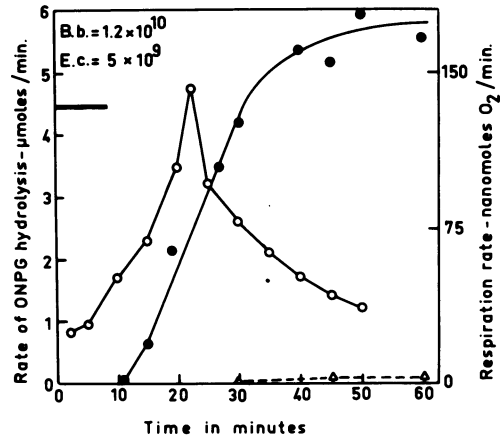

FIG. 7. *Unmasking of β-galactosidase activity during an infection cycle of B. bacteriovorus growing on a lactose cryptic E. coli. Hosts and parasites were mixed and samples of the total system (●), or of the supernatant fluid after sedimenting cells (△), were assayed at the indicated times for their rates of ONPG hydrolysis. Symbol: ○, lactose respiration pattern of the same host-parasite system. The horizontal bar indicates the rate of ONPG hydrolysis by a suspension of 5 × 10⁹ uninfected E. coli disrupted by sonic oscillation; a suspension of 1.2 × 10¹⁰ bdellovibrios similarly disrupted hydrolyzed ONPG at a rate of 7 nmoles per min.*

cause of the observed decrease in respiration rate, subject only to the assumption that cells permeable to ONPG are concomitantly permeable to lactose. This assumption was verified by demonstrating, after the peak rate of lactose respiration was attained, the formation of glucose from lactose by the host-parasite system. The observation that the rate of hydrolysis of ONPG, and hence its rate of permeation, continues to increase after the rate of lactose respiration starts to decline, supports the suggestion that the observed lactose respiration rate at any time results from two effects occurring concurrently, increasing permeability and decreasing respiratory potential.

Figure 8 shows the effect of multiplicity of infection at constant host population on the changes in rate of ONPG hydrolysis with time after infection. Both the rate of unmasking of β-galactosidase activity and the maximal rate of activity attained are directly proportional to the multiplicity of infection, over a range of multiplicities from 0.5 to 1.2. The relation between the maximal activity attained and the multiplicity of infection over a wider range is shown in Fig. 9. This plot assumes that the maximal rate of ONPG hydrolysis observed at a multiplicity of 4 represents a total unmasking of the β-galactosidase activity of all the host cells in the system, i.e., all hosts infected and sufficiently permeable so that enzyme is rate limiting. The percentage of β-galactosidase activity expressed at multiplicities of less than 4 is then a direct measure of the

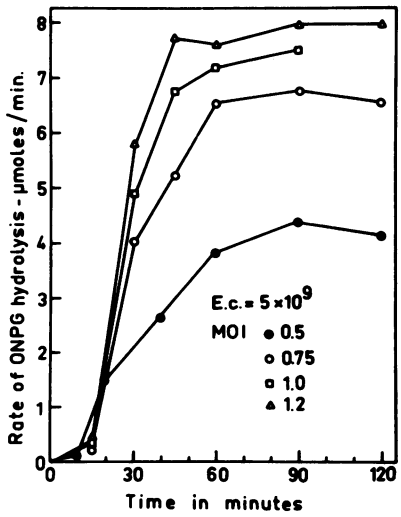

FIG. 8. *Effect of MOI on the unmasking of β-galactosidase activity. Experimental procedure as in Fig. 7; E. coli 5 × 10⁹ and bdellovibrios to MOI of 0.5 (●), 0.75 (○), 1.0 (□) and 1.2 (△).*

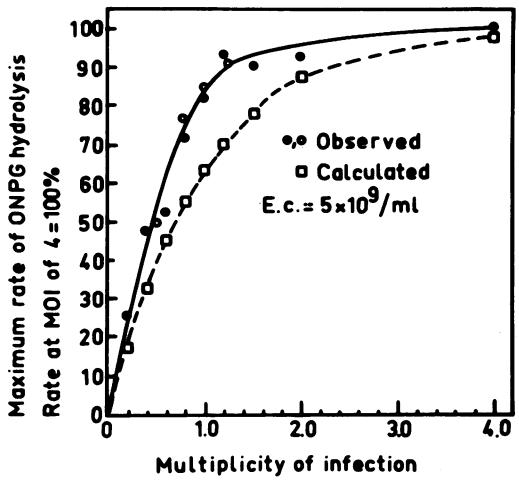

FIG. 9. *Per cent of total potential β-galactosidase activity of E. coli suspensions unmasked as a function of MOI. The maximal rate of ONPG hydrolysis observed at a MOI of 4 is taken as 100%. Experimental procedures as in Fig. 7. Symbols: ●, ○, experimental values; □, calculated values assuming a Poisson distribution of infected and noninfected E. coli over indicated range of MOI.*

percentage of the total host population infected. The results show that some 90% of the hosts are infected at multiplicities of 1.2 to 1.5. This is considerably higher than that predicted if the distribution of infected and noninfected hosts at different multiplicities followed a Poisson distribution (Fig. 9, calculated curve), as is the case in phage infection of *E. coli* (2).

Effect of inhibitors on early damage. Varon and Shilo (15) showed (i) that sodium chloride (0.9%) and phosphate (0.05%), among other compounds, prevent attachment of *B. bacteriovorus* strain 109 to its hosts; (ii) that streptomycin and other inhibitors of protein biosynthesis prevent penetration of bdellovibrio into its host without preventing its attachment; and (iii) that penicillin permits both attachment and penetration of bdellovibrio but not its growth.

These compounds were tested for their effects on early damage to permeability control and respiration of the host. No host damage could be detected in host-parasite mixtures when attachment of the parasite was prevented by the addition of either sodium chloride or phosphate to the system.

In the presence of 250 μg of streptomycin per ml, attachment was rapid but penetration could not be detected by phase microscopy even after several hours. The effect of streptomycin at this concentration on the unmasking of β-galactosidase activity is shown in Fig. 10. The earlier

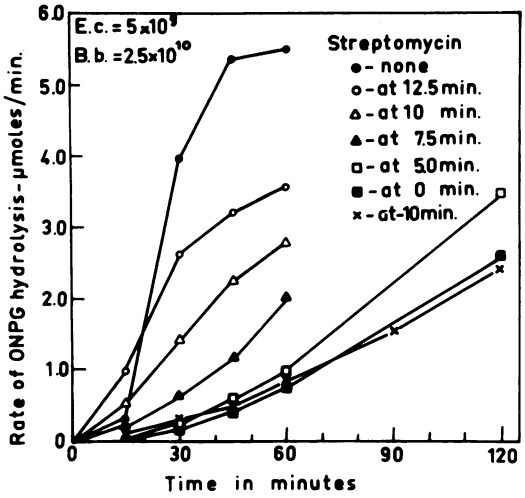

FIG. 10. *Effect of streptomycin (250 μg/ml) on the unmasking of β-galactosidase activity. Streptomycin was added to system [or to parasite suspension (×)] at indicated times after mixing host and parasite suspensions and rates of ONPG hydrolysis were determined at intervals on samples of the mixtures.*

streptomycin is added to the system, the slower the process. However, even preincubation of the parasite for 10 min in streptomycin before adding host did not prevent a change in permeability of the host from occurring. Incubating uninfected hosts with streptomycin did not result in a detectable change in permeability to ONPG over a 2-hr period.

Puromycin and chloramphenicol had effects similar to that of streptomycin. Penicillin had little or no effect on the development of host permeability during infection.

The effect of streptomycin on the decay of succinate respiration was similar to its effect on the development of permeability. The earlier its addition to the host-parasite system, the slower the decay of the succinate respiration rate (Table 4). Nevertheless, streptomycin addition even at zero time did not prevent the process.

Effects of dilute nutrients, Ca^{++}, and Mg^{++}. Since all experiments so far described were done in Tris buffer, the question arises whether the changes observed would occur in environments perhaps more favorable for maintaining host integrity. The basic experiments were repeated in DNB, a dilute nutrient medium typically used for propagation of bdellovibrio on its host, and in the presence of calcium and magnesium ions. The latter control was particularly important since Seidler and Starr (7) reported that, in the absence of these cations, *E. coli* infected by bdellovibrio undergoes a premature lysis. The data show that the development of permeability to ONPG is, if anything, slightly more rapid in the presence of DNB or the cations (Fig. 11 and 12). Likewise, the decay of the succinate respiration rate is not appreciably influenced by the addition of cations to the environment (Fig. 13). The changes, therefore, are not a consequence of the use of unsupplemented Tris buffer as the menstrum in the experiments reported.

DISCUSSION

The data show that one early consequence of the infection of a host by bdellovibrio is a rapid change in the permeability of the host to certain small molecules. Although the evidence on this point is largely confined to changes in permeability of *E. coli* ML 35 to lactose or ONPG, fragmentary data also indicate an increased

TABLE 4. *Effect of the time of streptomycin addition on the decay of succinate respiration during growth of B. bacteriovorus strain 109 on E. coli ML35*[a]

Time of streptomycin addition (min)	Rate of succinate respiration at 30 min (per cent of initial rate)
0	70
2	68
5.5	50
10.5	40
None	38

[a] System: 4×10^9 *E. coli*, 10^{10} *B. bacteriovorus*, 10 μmoles of succinate and 500 μg of streptomycin in 2.1 ml of 0.001 M Tris, *p*H 7.5, at 30 C.

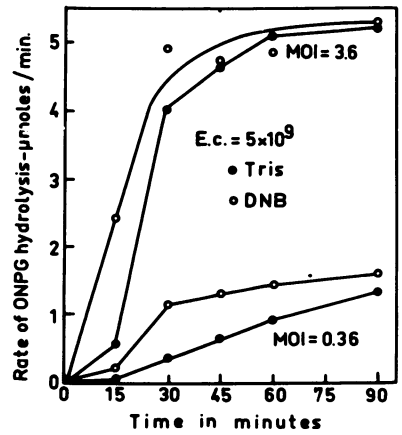

FIG. 11. *Effect of "dilute nutrient broth" on the unmasking of β-galactosidase activity during growth of B. bacteriovorus on E. coli. E. coli and bdellovibrio suspensions were prepared in DNB (○) or in Tris buffer, pH 7.5 (●), and mixed, and samples of the mixtures were assayed at intervals for their rates of ONPG hydrolysis.*

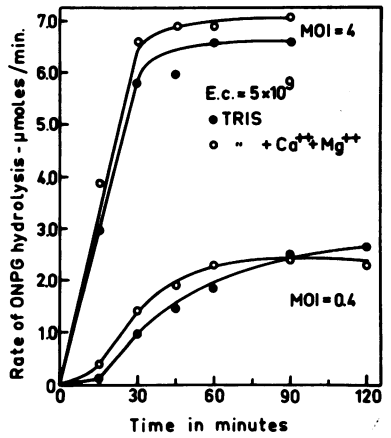

FIG. 12. *Effect of cations on the unmasking of β-galactosidase activity during growth of B. bacteriovorus on E. coli. Experimental procedure as in Fig. 11, except that cell suspensions were prepared in Tris buffer (●) or in Tris buffer containing 0.002 M Ca^{++} and 0.003 M Mg^{++} (○).*

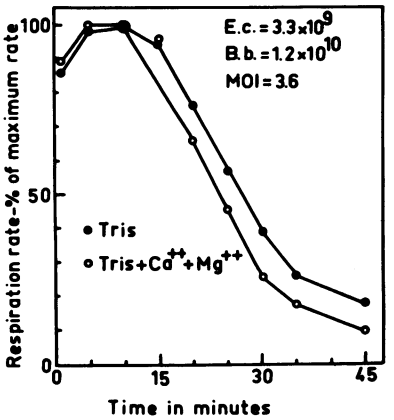

FIG. 13. *Effect of cations on the succinate respiration pattern during growth of B. bacteriovorus on E. coli. Host and parasite suspensions, prepared in Tris buffer (●) or in Tris buffer containing 0.002 M Ca^{++} and 0.003 M Mg^{++} (○), plus 10 μmoles of succinate were mixed and respiration rates were determined at intervals.*

permeability to acetate (Table 3), and to succinate (Fig. 13) in *E. coli* ML 35 and in *E. coli* B (*see* introduction) early in infection. Consequently, the effect could well be general. Since it is accepted that the permeability barrier of the eubacteria is the cytoplasmic membrane (6), it may be concluded that this structure is damaged early in infection.

The data also show a rapid decrease in respiratory potential of the infected host as measured with a variety of substrates. Here, two general alternatives must be considered; either a component specific to the metabolism of each of the compounds tested is damaged, or else some key step(s) common to all host respiration is being disrupted. The data favor the second interpretation. First, it seems unlikely that the respiration of six compounds chosen at random would show essentially the same decay rate unless some step common to the respiration of all were affected. Second, there is no evidence for rapid destruction of host enzymes either from the β-galactosidase activity measurements made in this study or from studies on release of radioactivity from ^{14}C-labeled hosts (E. Druker, M.S. Thesis, Hebrew University-Hadassah Medical School, 1969). Third, and most important, the decrease in potential to respire succinate or malate, components of the tricarboxylic acid cycle, must involve elements of either the cycle or the electron transport system and thus must involve elements common to all aerobic respiration in the host.

It is known that components of the electron transport chain and of the tricarboxylic acid cycle are localized in the cytoplasmic membrane (5), and it may be assumed from studies of mitochondria (3) that these elements are present in some structurally organized relation to each other. The changes in permeability and in respiratory function of the host early in infection may thus be due to a common process involving membrane damage. This conclusion is strengthened by the observation that streptomycin influences the rate of change in permeability and in respiration in a similar manner.

The striking differences in the effects of infection on lactose and succinate respiration of the host do not argue against a common process of damage. The host cells employed had essentially maximal potential for succinate respiration at the time of infection, and only a decrease in its rate of respiration of this compound would be possible. On the other hand, the host, lacking lactose permease, had essentially no potential for lactose respiration at the time of infection. Any time-dependent change that concurrently renders the host permeable and damages its respiratory potential would result in an increase in the rate of lactose respiration until the time when the rate of diffusion of lactose is sufficient to saturate the residual respiratory apparatus with substrate. Beyond this time in the infection cycle, further damage to the respiratory apparatus would result in a decreased rate of lactose respiration despite an increased rate of diffusion through a progressively more damaged membrane.

The data give no clue as to how membrane damage is effected. It is known that certain

strains of bdellovibrio produce an exoprotease which can lyse heat-killed host cells (8), but no such enzyme has yet been detected in strain 109. In fact, it is not known whether host damage is an autolytic process triggered by bdellovibrio infection or is due to enzymes of the parasite. Part of the data presented here support the latter alternative. The decay of succinate respiration is linear with time over much of the process, and the decay curves observed are what would be predicted if a fixed amount of enzyme were acting on a limited amount of substrate initially in excess, i.e., a membrane component. Since the rate of decay of succinate respiration and the rate of unmasking of β-galactosidase at a fixed host population increase with increasing multiplicities of infection beyond the numbers of bdellovibrios required to infect all hosts, these rates are apparently a function of the numbers of bdellovibrios attached to a host. Hence, the bdellovibrio and not the host is the probable source of the factor producing damage. If the factor is an enzyme, it probably pre-exists in the bdellovibrio before infection since agents that interfere with protein synthesis do not completely prevent the damage.

The rapidity of inception of these changes in the host combined with the observation that they occur in the presence of agents that prevent "penetration" of the parasite makes it certain that the entire parasite need not enter into the host periplast before damage is done to the membrane. However, as has been shown by the electron microscope studies of Burnham et al. (1), penetration involves a series of complex changes both in the host wall and in the parasite. The measurements of Varon and Shilo (15) show only that streptomycin and other agents prevent the consummation of the penetration process, but do not show at which stage the process is blocked. Since the rate of membrane damage increases with time after infection when streptomycin is added to the system very early (-10 to $+7.5$ min; *see* Fig. 10), some aspect of the infection cycle beyond initial attachment apparently continues in the presence of streptomycin. An electron microscope study of the streptomycin-inhibited cycle should prove of interest in this connection.

It was observed that agents that prevent attachment of bdellovibrio to its host also prevent membrane damage. This suggests either that an exoenzyme is not involved or else, if it is, that it cannot reach the membrane without previous damage to the host wall. Shilo (8) suggested that a contact enzyme, similar to the cellulase of the cytophaga (11), may be responsible for initiating host damage. Another possibility is that upon attachment an enzyme is excreted or even injected by the bdellovibrio through the host wall to act on the host membrane.

Although the details remain to be resolved, the general picture that emerges from these studies is that, after attachment and even before complete penetration of the bdellovibrio, the host membrane is damaged in such a way as to decrease host respiration and permit free diffusion of small molecules into (and out of; *see* Druker, M.S. Thesis, The Hebrew University-Hadassah Medical School, 1969) the host protoplast. This picture differs from that based on electron microscopy observations which do not in general show early damage to the cytoplasmic membrane. It has been pointed out, however, that such damage must occur before the bdellovibrio can derive nutrients from the host protoplast (8).

The observed respiration pattern of the unsupplemented host-parasite system also permits some conclusions about the infection cycle. *B. bacteriovorus* strain 109 has an unusually high endogenous respiration, some seven times that of *E. coli* on an equivalent protein basis. This may not be typical of all strains of bdellovibrio since Simpson and Robinson (10) report an endogenous rate of 20×10^{-12} μliters of O_2 per cell per hr for strain 6-5-S, which is less than 0.01 the rate of strain 109 (0.15 versus 18 nmoles per min per 10^{10} cells). Of the compounds tested, only the complex mixtures peptone, yeast extract, and casein hydrolysate stimulated the respiration of strain 109, and the effect was small. It cannot be concluded that this strain, either free or in the host, is incapable of respiring exogenously supplied compounds such as succinate. It may well be that the free parasite is respiring endogenous reserves at a rate that nearly saturates its respiratory potential. Thus any respiration of exogenously supplied substrates could be masked by a corresponding decrease in the rate of respiration of endogenous materials. This possibility can be checked by the use of either labeled substrate or prelabeled parasites.

The increase in endogenous respiration rate over the first 15 min of the infection cycle cannot be attributed with certainty to a change in the respiration of the parasite, the host, or both. Conceivably, this increase may be another facet of host membrane damage, e.g. an early uncoupling of respiratory control, but this is only a speculation. Alternatively, it may be a consequence of nutrients from the host becoming available to the attacking parasite since the increase is of the same magnitude as the stimulation of respiration of the free bdellovibrio by complex substrates.

The second rise in respiration rate of the unsupplemented system that starts about 1 hr after initiation of the cycle (Fig. 1 and 2) is clearly a consequence of increased bdellovibrio respiration since by this stage in the cycle host respiration has been largely eliminated. This second rise is gradual, not sudden as occurs when a substrate is added to a suspension of typical eubacteria in a nonnutrient environment, and the rise continues until the onset of host lysis. The respiration rate attains a level some three to four times that of the endogenous respiration rate of the initial bdellovibrio population. This increase is about the same as the average increase in plaque-forming units during the cycle. It appears, therefore, that the rise is in large measure a consequence of the intracellular growth of the parasite. If so, the intracellular phase of infection can be divided into at least two stages, preparation for growth lasting to about 60 min under the conditions of these experiments, followed by growth per se. This conclusion should be checked by direct measurements of bdellovibrio mass increase during the intracellular phase. A second conclusion arising from the same data is that bdellovibrios not only have a complete energy-generating system as was shown by Simpson and Robinson (10) but also that their system is the exclusive source of energy for intracellular growth.

The observation that permeability damage at low multiplicities of infection is greater than that predicted if the distribution of attached bdellovibrios followed the Poisson equation suggests that the bdellovibrios may have a search mechanism for uninfected hosts. This conclusion is tentative since the degree of uncertainty in the enumeration of hosts and parasites is possibly as great as the observed divergence from the Poisson distribution. However, it is known that bdellovibrios may reversibly attach to a host (12). The basis of reversible attachment is not known but could be due in part to the limited number of effective attachment sites which may vary from a few or none to many on individual host cells (8). Regardless of the basis, the fact that some bdellovibrios, after attachment to a host, may leave that host and attach to another provides a partial explanation for the departure from the Poisson distribution. This phenomenon in essence increases the effective multiplicity of infection.

ACKNOWLEDGMENTS

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